PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Eric ADRIAENSSENS et al. Group Art Unit: 1642

Application No.: 10/530,568 Examiner: M. HALVORSON

Filed: April 7, 2005 Docket No.: 123439

For: METHOD FOR NGF ASSAY FOR IN VITRO DIAGNOSIS OF BREAST CANCER AND THERAPEUTIC USE

DECLARATION UNDER 37 C.F.R. §1.132

I, Dr. Genevieve Choquet-Kastylevsky, a citizen of France, hereby declare and state:

1. I have a degree in Medicine (Dermatology and Immunology) that was conferred upon me by Lyon and Paris Universities in 1996, a Ph.D. in Immunology (Lyon University, 2001), and Master's Degrees in Molecular Biology (Paris University, 1990) and Pharmacology (Lyon University, 1997).

2. I have been employed by bioMerieux since 2001 and I have had a total of 8 years of work and research experience in diagnostic assay technology.

3. I am a member of the European Association of Cancer Research, and of the French Proteomic Society (SFEAP).

4. My publications include the following works in this field: Alix-Panabieres et al., Immunol Methods., 2005; Canelle et al., Electrophoresis, 2006; Perronet et al., Proteomics, 2006; Caron et al., Mol. Cell Proteomics, 2007; and Morla et al., Electrophoresis (currently under publication). My public appearances include speaking engagements on proteomics and cancer before attendees to international symposiums.

5. I am a named inventor in the above-captioned patent application.

6. I am employed by one of the co-assignees of the above-identified patent application. In the course of that professional relationship, I receive compensation from that co-assignee for my work. I am not being separately compensated for my work in connection with this Declaration.

7. Nerve growth factor (NGF) is the prototypic member of the Neurotrophin family of growth factors. The neurotrophin family includes NGF, Brain-derived neurotrophic factor (BDNF), NT-3, and NT4/5, which share 50% of sequence homology. In terms of biological activities, the members of the neurotrophin family are well described for their stimulating effect on the survival and differentiation of different sub-populations of neurons during development, homeostasis, and regeneration of the nervous system. The receptors of the neurotrophins are TrkA (bind NGF), TrkB (bind BDNF and NT4/5), and TrkC (bind TrkC) -- they are all membrane receptors that exhibit a tyrosine kinase activity upon ligand stimulation. A second category of receptors is represented by p75NTR, which is able to bind all neurotrophins and appears to have a regulatory effect on the activity of the Trks.

8. In the experiment described below, conducted under the common supervision and review of Professor Hubert Hondermark and me, we have recently shown that NT4/5 and BDNF are expressed and released by breast cancer cells. This has been shown both in vitro, by analyzing cell lines in tissue culture, and in vivo, by analyzing breast tumor biopsies. Conversely, NT-3 mRNA, detected at 249 pb was less or not expressed, depending on cell types (Fig A), nor detected by Western Blot (Fig C). In vitro, human breast epithelial cell lines (MCF-7, T-47D, MDA-MB-231, BT-20, and MCF-10A) expressed the BDNF and NT-4/5 transcripts, visualized at 200 pb and 164 pb respectively after RT-PCR (Fig. A).

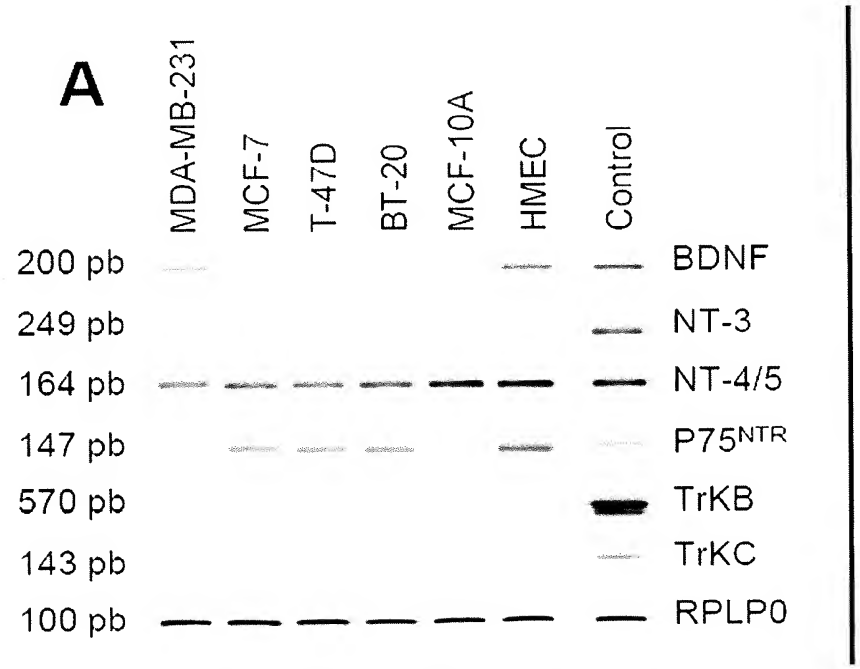

Fig. A: Expression of neurotrophins and their receptors in breast cancer cells in vitro. Total RNA from breast cancer cell lines (MDA-MB-231, MCF-7, T-47D, BT-20) and normal breast epithelial cells (MCF-10A and HMEC), plus NT2/D1 as control, were isolated and reverse-transcribed. Real time PCR amplifications of BDNF, NT-3, NT-4/5, p75NTR, TrkB, TrkC, and RPLP0 as loading control, were migrated in 2 % agarose gel.

9. Moreover, a western-blotting on total cell lysates revealed 14 kDa bands corresponding to BDNF and NT-4/5 in all cell types tested (Fig. C).

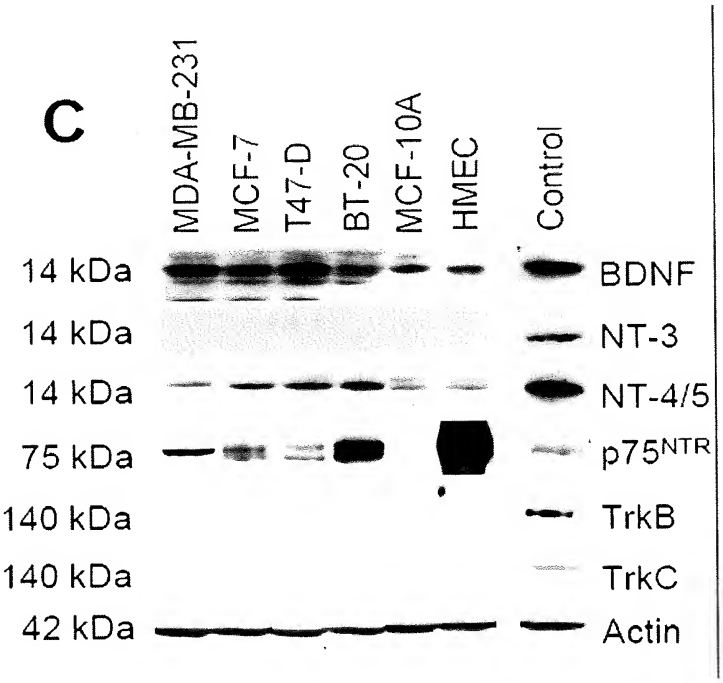

Fig. C : Protein extracts of various cells were processed for Western blotting using anti-neurotrophins (anti-BDNF, anti-NT-3, anti-NT-4/5) and anti-receptors (anti-p75NTR, anti-TrkB, anti-TrkC) antibodies. Recombinant proteins or NT2/D1 cells served as positive control and an anti-actin antibody was used for equiloading control.

10. In addition, results presented in Fig. D show that BDNF and NT-4/5 were detected in conditioned media from both normal and breast cancer cells, strongly suggesting that these neurotrophins are secreted by breast epithelial cells. Conversely, NT-3 mRNA, detected at 249 pb was less or not expressed, depending on cell types (Fig. A), nor detected by Western Blot (Fig. C).

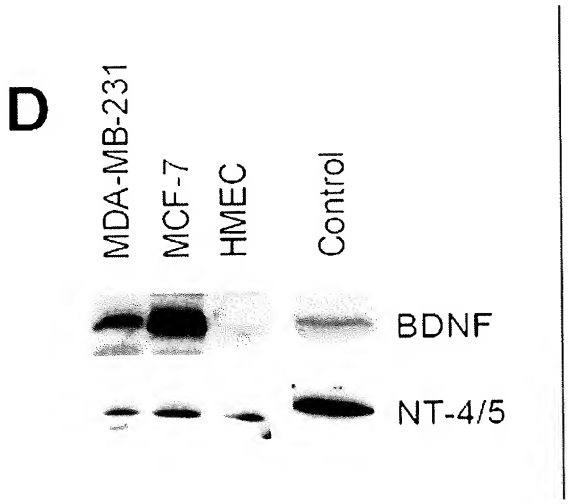

Fig. D: Secretion of BDNF and NT-4/5 by breast epithelial cells. Immunoblotting of conditioned media from MDA-MB-231, MCF-7 cells and HMEC were performed using anti-BDNF and anti-NT-4/5 antibodies, recombinant BDNF and NT-4/5 proteins served as control.

11. To study the distribution of BDNF and NT-4/5 in vivo, breast tissue arrays corresponding to 45 breast tumor samples were analysed by immunohistochemistry. Fig. E exemplifies the expression of BDNF and NT4/5 in breast tumor from human patients, compared to normal mammary tissue. The relative quantification of immunostaining intensities is presented in Table 1.

Fig. E

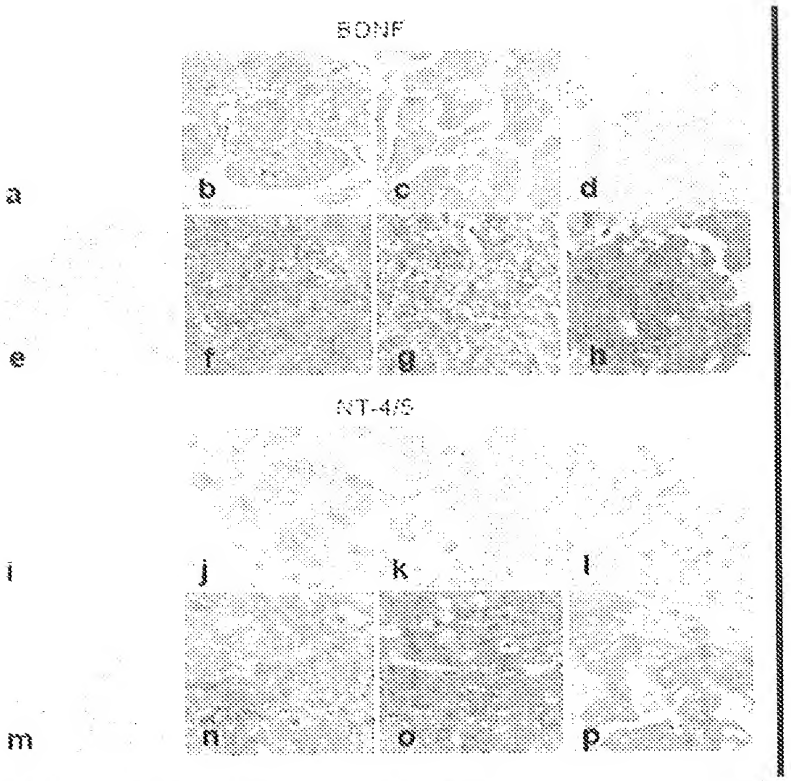

Fig. E: Immunohistochemistry of BDNF and NT-4/5 in breast tumors. Antibodies against BDNF and NT-4/5 were used on breast tissue arrays. Specific immunoreactivity was observed for all histological types of breast cancer compared to normal breast tissues (e, m) or control without primary antibody (a, i). b, j: ductal carcinoma *in situ*; c, k: infiltrating ductal carcinoma; d, l: infiltrating lobular carcinoma; f, n: metastatic atypical medullary carcinoma in lymph node; g, o: metastatic infiltrating ductal carcinoma in lymph node; h, p: metastatic infiltrating lobular carcinoma in lymph node. Relative quantification of immunostaining intensities are presented in Table 1.

Table 1

Clinicopathological factors		NT-4/5						BDNF					
			labelling intensity				Mean	SD		labelling intensity				Mean	SD
			0	1	2	3				0	1	2	3		
Breast Normal tissues		(n=9)	1	4	4	0	1.3	0.7	(n=9)	1	4	2	2	1.6	1.0
Breast Cancer tissues		(n=35)	2	5	11	17	2.2	0.9	(n=36)	3	10	13	10	1.8	0.9
Breast cancer metastatic tissues (lymph node)		(n=9)	0	2	5	2	2.0	1.0	(n=9)	0	1	4	4	2.3	0.7
cancer – metastase		(n=44)	2	7	16	19	2.2	0.9	(n=45)	3	11	17	14	1.9	0.9
Stage	0	(n=2)	0	0	0	2	3.0	0.0	(n=2)	0	0	2	0	2.0	0.0
	I	(n=0)	0	0	0	0	0.0	0.0	(n=0)	0	0	0	0	0.0	0.0
	II	(n=15)	0	2	5	8	2.4	0.7	(n=15)	1	2	4	8	2.3	0.9
	III	(n=27)	2	5	11	9	2.0	0.9	(n=28)	2	9	11	6	1.8	0.9
Estrogen receptors	–	(n=25)	2	4	7	12	2.2	1.0	(n=25)	3	4	9	9	2.0	1.0
	+	(n=19)	0	3	9	7	2.2	0.7	(n=20)	0	7	8	5	1.9	0.8
Progesterone receptors	–	(n=18)	1	2	6	9	2.3	0.9	(n=18)	2	1	7	8	2.2	1.0
	+	(n=25)	1	5	10	9	2.1	0.8	(n=26)	1	10	10	5	1.7	0.8
Lymph nodes status	–	(n=32)	2	5	12	13	2.1	0.9	(n=33)	2	10	11	10	1.9	0.9
	+	(n=12)	1	2	3	6	2.2	1.0	(n=11)	1	1	5	4	2.1	0.9
P53	–	(n=18)	1	2	8	7	2.2	0.8	(n=17)	1	6	4	6	1.9	1.0
	+	(n=26)	1	5	8	12	2.2	0.9	(n=28)	2	5	13	8	2.0	0.9

Table 1: NT-4/5 and BDNF expression compared to clinicopathological factors. The intensity of anti-NT-4/5 and anti-BDNF immunohistological staining in breast tumor biopsies (Fig. 1) was estimated from 0 (no staining) to 3 (intense staining) by two independent experimentators. For a few patients, some clinicopathological evaluations failed, and consequently, the total number of cases can differ from 44 and 45 for NT-4/5 or BDNF. For each tissue sample, the following clinicopathological information was obtained: stage (from the TNM classification of the UICC; ranging from 0 to III), hormone receptor status (estrogen receptor and progesterone receptor), lymph node and p53 status.

12. Of major interest here, we have shown that despite this production and release of BDNF and NT4/5 by breast cancer cells, these neurotrophins are not detected in the plasma of breast cancer patient (Fig. F). This clearly demonstrates that the production and release of a neurotrophin by breast cancer cells does not imply that this neurotrophin will be detected in the plasma.

Fig. F

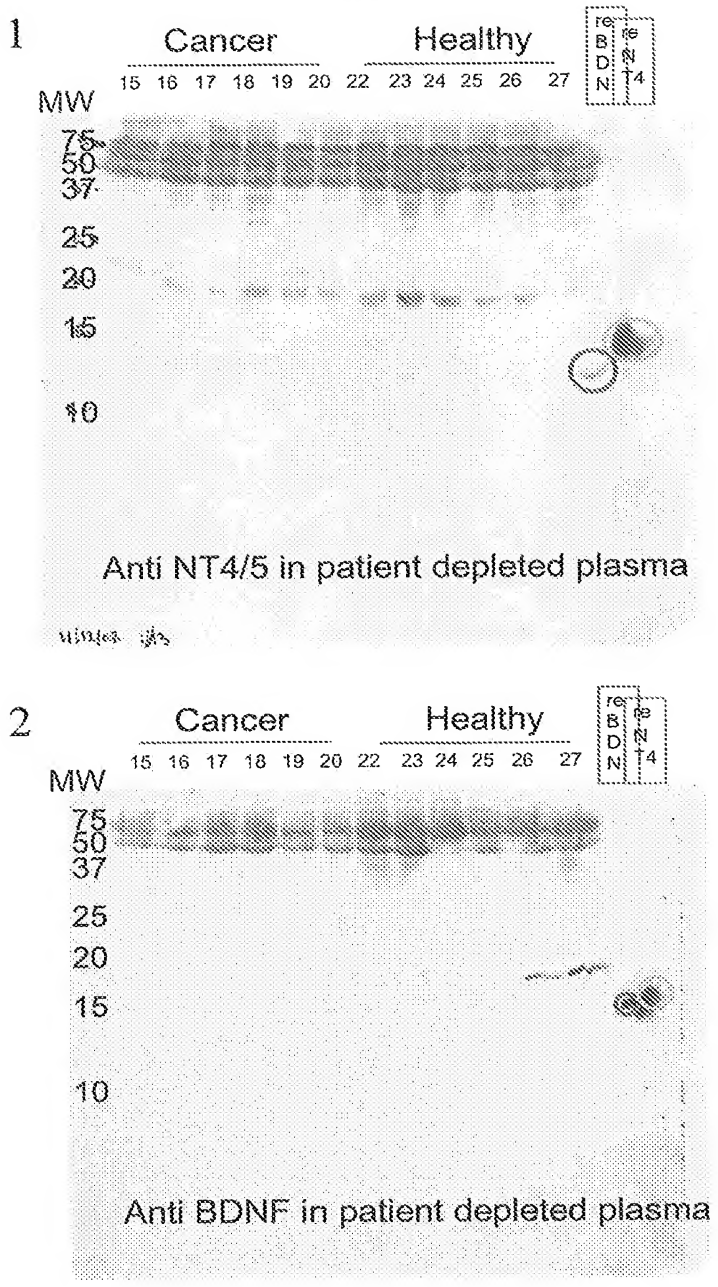

Fig. F: Western Blot revealed using anti NT4/5 antibody (1) or an anti BDNF antibody (2). The patients' plasma were loaded on the gel after a depletion to remove the more abundant proteins, and to allow the detection of low abundance proteins.

13. We conducted the following protocols to obtain the above results (Fig. F):

14. Protocol for plasma depletion (removal of major abundant proteins): Biological samples, stored at -80°C, were thawed on ice. 14 µL were taken and depleted (5188-6408, Agilent) according to the recommendations of Agilent. Once mixed F1 and F2 fractions were concentrated on Amicon Ultra-4 Ultracel 10 k (UFC801096, Millipore).

15. Protocol for SDS-PAGE of plasma samples: Depleted plasma's concentrate are thawed on ice, mixed with 5x laëmmli (5% SDS, 5% β-mercapto-éthanol, 50% glycérol, 50 mM Tris pH 6,8, 0,3% bromophénol blue) to 1x and boiled for 5 min at 95°C. Half of the volume concentrate+laëmmli (equivalent to 7 µL of plasma from patient) is loaded and separated by SDS-PAGE (Sodium DodecylSulfate-PolyAcrylamide Gel Electrophoresis) on a 12.5% acrylamide gel. Proteins are then transferred to nitrocellulose.

16. Protocol for Western-blot on plasma sample: After saturation in TBS (Tris Buffered Saline) (150 mM NaCl, 20 mMTris), 0.1% Tween20 pH 7.4 containing 5% Bovine serum albumine (60 min, RT), membranes were incubated with 1/1000, anti-NT4 antibody (SC-545, Santa cruz biotechnologies) in saturation (over night, 4°C). Then the membranes were rinsed with TBS 0.1% Tween20, incubated with 1/20 000 peroxidase–conjugated anti-rabbit antibody (711-035-152, Jackson ImmunoResearch) in saturation (60 min, RT). After extensive washes, the reaction was revealed using the Super Signal West Pico chemoluminescent substrat (Pierce Interchim, France) with Kodak X-Omat AR film.

17. Material used: Anti-NT4 (sc-545) and Anti-BDNF (sc-546) antibodies were used. Patient samples were used as represented below in Fig. G.

Fig. G

Plasma samples	
GCRL072/F0 BP 2A	15
GCRL080/F0 BP 2A	16
GCRL082/F0 BP 2A	17
GCRL079/F0 BP 2A	18
GCRL088/F0 BP 2A	19
GCRL091/F0 BP 2A	20
N836280/F0 HP 1A	22
N836213/F0 HP 1A	23
N836504/F0 HP 1A	24
N836483/F0 HP 1A	25
N836467/F0 HP 1A	26
N744275/F0 HP 1A	27

: stage I
: stage II
: stage III
: Healthy (age-matched blood donor)

18. As to the application of the Pica and Bigazzi references to the present application, medullary carcinomas of the thyroid and Kaposi sarcomas are very special cancers, totally unrelated to breast adenocarcinomas. Therefore, the findings of NGF in these two cancer types cannot be extrapolated to other cancer types, especially not to breast adenocarcinomas. Indeed, mammary carcinomas or mammary adenocarcinomas (the most frequent being galactophoric carcinomas and lobular carcinomas) are hormone-dependent carcinomas, which are of a "pure" epithelial nature and which are exceptionally muco-secreting. Medullary thyroid carcinomas are classified among neuroendocrine tumours and mostly produce calcitonin. Even if they express some cytokeratines, they are classified in a special group of carcinomas: the group of the neuroendocrine carcinomas that also includes neuroendocrine tumours present in other organs such as the lung. Kaposi sarcoma is a malignant mesenchymatous tumour made of a proliferation of spindle-shaped cells and neoplasic endothelial cells. These conjunctive tumours arise in an endemic way or either in

case of immunosuppression, in particular linked to AIDS, and are induced by an oncogenic virus, HHV8.

19. All statements made herein of my own knowledge are true, and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing there from.

Date: **April 21, 2009**

Genevieve Choquet-Kastylevsky

/Genevieve Choquet-Kastylevsky/